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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: SERINE PROTEASE AND TOPICAL RETINOID COMPOSITIONS (57) Abstract This invention is related to methods for treating Acne Vulgaris and/or for producing anti-aging effects on the skin of a mammal, and compositions effective for the same. More specifically, the present invention is directed to the use of serine proteases, as the sole active in a composition effective for the treatment of Acne Vulgaris and/or for producing anti-aging effects on the skin of a mammal, or in combination with a retinoid compound in a composition effective for the same.		

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-1-

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SERINE PROTEASE AND TOPICAL RETINOID COMPOSITIONS

Cross-Reference to Related Application

10 This Application claims the benefit of United States Provisional Application Number 60/037,605 filed on 12 February 1997, which is incorporated herein by reference in its entirety.

15 Field of the Invention

 This invention is related to methods for treating Acne Vulgaris and/or for producing anti-aging effects on the skin of a mammal, and compositions effective for the same. More specifically, the present invention is
20 directed to the use of serine proteases, either alone or in combination with a retinoid compound in a pharmaceutical or cosmetic composition.

Background of the Invention

25 Acne Vulgaris is a disorder of the pilosebaceous unit that affects nearly all adolescents to some degree, as well as many adults. The initial lesion of the disease is believed to be due to hypercornification and hyperkeratinization of the infundibulum, a process that
30 helps to transform the sebaceous follicle into a comedone. This disorganization of the epithelium may give rise to inflammatory lesions, as the infundibulum ruptures and sebum is introduced into the dermis.

-2-

5 Accordingly, traditional therapies are directed
against the three major pathological processes which
contribute to the development of Acne Vulgaris.
Treatments such as topical retinoids work against the
10 obstruction of the sebaceous follicle resulting from
abnormal desquamation of the follicular epithelium.
Hormonal agents target the androgen-stimulated increase
in the production of sebum. Finally, antibiotics
function to reduce and/or halt the proliferation of
propionibacteria within the follicle which contribute to
15 inflammation. Benzoyl peroxide, salicylic acid, and
various cleansing agents are also employed for similar
purposes. Topical retinoids are considered to be one of
the most effective classes of comedolytic agents for the
treatment of Acne Vulgaris, however their clinical
20 efficacy is limited by their irritant effects.

Topical retinoids have also been used to produce
anti-aging effects on the surface of mammalian skin.
While they are known in the art as one of the most
effective topical treatments available, these compounds
25 are limited by their irritant effects.

It would be desirable to provide a method for
treating Acne Vulgaris which is as effective as
traditional acne therapies, but which is not associated
with high levels of irritancy.

30 It would also be desirable to provide a method for
producing anti-aging effects on the surface of mammalian
skin which is as effective as retinoid treatments, but
does not have the same irritant effects.

-3-

5 Summary of the Invention

 In accordance with the present invention, we have found a method for treating Acne Vulgaris and/or for producing anti-aging effects on the skin of a mammal comprising, consisting essentially of, or consisting of
10 topically applying to the skin of a mammal an effective amount of a first topically active agent comprising a protease.

 In another embodiment of the present invention, we have found a method for treating Acne Vulgaris and/or for producing anti-aging effects on the skin of a mammal
15 comprising, consisting essentially of, or consisting of topically applying to the skin of a mammal an effective amount of a first topically active agent comprising a protease in combination with a second topically active
20 agent comprising a retinoid.

 In yet another embodiment of the present invention, we have found a pharmaceutical or cosmetic composition comprising, consisting essentially of, or consisting of:

25 a) a first topically active agent comprising a protease; and

 b) a second topically active agent comprising a retinoid.

 The compositions and methods of this invention provide a unique, convenient means for treating Acne
30 Vulgaris and/or for producing anti-aging effects on the skin of a mammal.

Brief Description of the Drawings

-4-

5 The file of this patent contains several drawings executed in color. Copies of this patent with said color drawing will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

10 The invention will be more fully understood and further advantages will become apparent when reference is made to the following detailed description of the invention and the accompanying drawings in which:

15 FIG. 1(a) is a representation which illustrates a cross-sectional view of the skin of a Rhino mouse one hour after treatment with fluorescently labeled trypsin. FIG. 1(b) is a representation which illustrates a cross-sectional view of the skin of a Rhino mouse four hours after treatment with fluorescently labeled trypsin. FIG. 20 (c) is a representation which illustrates a cross-sectional view of the skin of a Rhino mouse four hours after treatment with fluorescently labeled trypsin, following 5 days of daily treatment with 1% (w/v) trypsin.

25 FIGS. 2(a) and 2(b) are representations which illustrate the histology of Rhino mouse skins processed with H&E staining, (a) untreated, and (b) treated daily with 0.1% (w/v) trypsin in GDL liposomes for five days.

30 FIGS. 3(a) and 3(b) are color representations which illustrate the cross-sectional view of Rhino mouse skins which were processed for paraffin sections and stained for elastin. FIG. 3(a) is vehicle treated, and FIG. 3(b) is trypsin treated. FIGS. 3(c) and 3(d) are representations which illustrate the cross-sectional

-5-

5 view of C57Bl/6 mouse skins which were processed for paraffin sections and stained for elastin. FIG. 3(c) is vehicle treated, and FIG. 3(d) is trypsin treated.

10 FIGS. 4(a-c) are representations which illustrate the histology of Rhino mouse skins processed with H&E staining, (a) treated with 0.0005% (w/v) all-trans retinoic acid, (b) treated with 0.005% (w/v) trypsin, and (c) treated with both 0.0005% (w/v) all-trans retinoic acid and 0.005% (w/v) trypsin.

15 FIGS. 5(a) and 5(b) are representations which illustrate the cross-sectional view of the TUNEL-stained skin tissue of a vehicle treated Rhino mouse. FIGS. 5(c) and 5(d) are representations which illustrate the cross-sectional view of the TUNEL-stained skin tissue of a Rhino mouse treated with trypsin.

20 FIG. 6 is a representation which illustrates the profile of gene expression of trypsin treated Rhino mouse skins at various concentrations of trypsin as detected by Reverse Transcription-Polymerase Chain Reaction ("RT-PCR").

25 Detailed Description of the Invention

As used herein "(w/v)" shall mean grams of a given component per 100 ml of the total composition.

30 Topically active agents suitable for use in the compositions of the present invention as the first topically active agent include proteases, which include, but are not limited to, serine proteases. Preferably, the first topically active agent is selected from trypsin, carboxypeptidase-Y, protease IV, subtilysin, or

-6-

5 mixtures thereof. The protease of choice is trypsin. Preferably, the protease is present in an amount, based upon the total volume of the composition of the present invention, of from about 0% (w/v) to about 5% (w/v), and more preferably from about 0.01% (w/v) to about 1%
10 (w/v).

While not wishing to be bound by any theory, it is believed that the first topically active agent of the present invention treats the hyperkeratinization associated with Acne Vulgaris and/or produces anti-aging effects on the skin. Though the first topically active agent can be used as the sole active ingredient in a composition for the treatment of Acne Vulgaris and/or to produce anti-aging effects on the skin, to more thoroughly treat Acne Vulgaris, the first topically active agent of the present invention can be combined with a second topically active agent.
15 20

Again, while not wishing to be bound by any theory, it is believed that said second topically active agent treats both the hyperkeratinization and the obstruction of the sebaceous follicle associated with Acne Vulgaris, while also producing anti-aging effects on the skin which are comparable to those produced by the first topically active agent. Thus, as evidenced by Example 6 herein, the first feature of combining said first and second topically active agents is that the resulting treatment attacks at least two of the pathological processes associated with Acne Vulgaris, while not sacrificing the anti-aging benefits of the first topically active agent.
25 30

-7-

5 A second feature of combining said first and second
topically active agents is evidenced by Example 4
herein, which shows that combining said first topically
active agent with said second topically active agent
mitigates the irritant effect associated with said
10 second topically active agent. Thus, the efficacy of
treatment of Acne Vulgaris and/or signs of anti-aging
effects on the skin are approximately the same with the
treatments of the present invention as compared with
treatments involving the second topically active agent
15 alone, but the irritant effect normally associated with
said second topically active agent is substantially
reduced.

 A third feature of combining said first and second
topically active agents is evidenced by Example 7
20 herein, which shows that combining said first topically
active agent with said second topically active agent
substantially reduces the time necessary for product
efficacy as compared to the use of the second topically
active agent alone. Thus, the efficacy of treatment
25 remains approximately the same as compared with
treatments utilizing the second topically active agent
alone, but the length of time required to see results
normally associated with said second topically active
agent is substantially reduced by combining said second
30 topically active agent with said first topically active
agent.

 Topically active agents suitable for use in the
compositions of the present invention as the second
topically active agent include those compounds in the

-8-

5 class of retinoids, which include, but are not limited
to, retinoic acids, vitamin A alcohol, vitamin A
aldehyde, retinyl acetate, retinyl palmitate, or other
derivatives, analogs or mixtures thereof. The retinoid
of choice is all-trans retinoic acid. Preferably, the
10 retinoid is present in an amount, based upon the total
volume of the composition of the present invention, of
from about 0.0001% (w/v) to about 0.5% (w/v), and more
preferably from about 0.001% (w/v) to about 0.025%
(w/v).

15 If the delivery parameters of the first topically
active agent so require, the pharmaceutical or cosmetic
compositions of the present invention may preferably be
further comprised of a pharmaceutically or cosmetically
acceptable vehicle capable of functioning as a delivery
20 system to enable the penetration of the topically active
agent into the utriculus. While any commercially
available vehicle for delivering the first topically
active agent to the appropriate skin appendage, which in
this case is the utriculus, is suitable for use as the
25 pharmaceutically or cosmetically acceptable vehicle,
liposomes are preferred. The liposomes are more
preferably non-ionic and comprised of: (a) glycerol
dilaurate or glycerol distearate; (b) compounds having
the steroid backbone found in cholesterol; and (c)
30 fatty acid ethers having from about 12 to about 18
carbon atoms, wherein the constituent compounds of the
liposomes are in a ratio of about 53:10:22 to about
63:20:32, and preferably from about 55:12:24 to about
61:18:30, respectively. Liposomes comprised of glycerol

-9-

5 dilaurate / cholesterol / polyoxyethylene-10-stearyl
ether ("GDL") are most preferred. Preferably the
liposomes are present in an amount, based upon the total
volume of the composition, of from about 10 mg/mL to
10 about 100 mg/mL, and more preferably from about 25 mg/mL
to about 50 mg/mL. A ratio of about 58:15:27,
respectively, is most preferred. Suitable liposomes
may preferably be prepared in accordance with the
protocol set forth in Example 2, though other methods
commonly used in the art are also acceptable.

15 The above described liposomal composition may be
prepared by combining the desired components in a suitable
container and mixing them under ambient conditions in any
conventional high shear mixing means well known in the art
for non-ionic liposomes preparations, such as those
20 disclosed in Niemiec et al., "Influence of Nonionic
Liposomal Composition On Topical Delivery of Peptide
Drugs Into Pilosebaceous Units: An *In Vivo* Study Using
the Hamster Ear Model," 12 Pharm. Res. 1184-88 (1995)
("Niemiec"), which is incorporated herein by reference
25 in its entirety.

30 In alternative embodiments, the pharmaceutical or
cosmetic composition of the present invention may be
optionally combined with other ingredients such as
moisturizers, cosmetic adjuvants, anti-oxidants,
surfactants, foaming agents, conditioners, humectants,
30 fragrances, viscosifiers, buffering agents, sunscreens,
colorants, preservatives, and the like in an amount
which will not destroy the liposomal structure, if

-10-

5 present, in order to produce cosmetic or pharmaceutical products.

10 When used in combination with one another, the first and second topically active agents of the present invention can be applied to the skin of a mammal either simultaneously or at different times. For example, in a first instance, if daily treatment with the combination of the first and second topically active agents is desired, the first topically active agent can be administered in the morning and the second topically active agent can be administered in the afternoon. In a second instance, to serve as an example only, the second topically active agent can be administered in the morning and the first topically active agent can be administered in the afternoon. In a third instance, again, to serve as an example only, the first and second topically active agents can be administered together. In a fourth instance, serving only as an example, the first and second topically active agents can be administered on alternate days. Furthermore, in a fifth instance, serving only as an example, the treatments with the first and second topically active agents do not have to be given in a one-to-one dosage, so the first topically active agent can be administered for two days, while the second topically active agent is administered on the third day and so on. There are, of course, multiple variations of this fifth instance. The previous five examples are provided only to illustrate some of the many different treatment regimens possible with the methods of the present invention. It should be

15
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-11-

5 understood that these examples are not limiting in any way to the treatment methods of the present invention, and that many other treatment regimens are possible.

10 The pharmaceutical or cosmetic composition should be applied in an amount effective to treat Acne Vulgaris and/or produce anti-aging effects on the skin. As used herein "amount effective" shall mean an amount
15 sufficient to cover the region of skin surface where treatment of Acne Vulgaris and/or production of anti-aging effects is desired. Preferably, the composition is applied to the skin surface such that, based upon a square cm of skin surface, from about 2 $\mu\text{l}/\text{cm}^2$ to about 8 $\mu\text{l}/\text{cm}^2$ of topically active agent is present when treatment of Acne Vulgaris and/or production of anti-aging effects on the skin is desired.

20 The invention illustratively disclosed herein suitably may be practiced in the absence of any component, ingredient, or step which is not specifically disclosed herein. Several examples are set forth below to further illustrate the nature of the invention and the manner of
25 carrying it out. However, the invention should not be considered as being limited to the details thereof.

Examples

EXAMPLE 1: The Rhino Mouse System

30 The Rhino mouse has been used as an experimental acne model to screen topically active comedolytic and antikeratinizing agents as described in Sundberg, J.P., "The Hairless and Rhino Mutations, Chromosome 14,"

-12-

5 Handbook of Mouse Mutations With Skin and Hair
Abnormalities 291-312 (1994), which is incorporated
herein by reference in its entirety. A recessive
mutation on chromosome 14 results in a mouse with
10 wrinkled skin devoid of body hair by age 25 days. At
that time, the end of the first hair cycle, the
follicular papillae fail to follow the regressing hair
follicles and become isolated in the dermis. The
papillae do not reassociate with the follicular
epithelium to initiate a new hair follicle cycle. The
15 upper remnants of the hair follicle are filled with
sloughed, cornified cells and form utriculi with a small
sebaceous gland at their base, resembling an open
comedone. The rhino skin becomes progressively loose,
forming folds and ridges, due to the expansion of the
20 surface, secondary to abortive hair follicles filling
with cornified debris. The utriculi progressively
enlarge, forming pilary cists (pseudocomedones), which
are dilated follicular infundibula filled with cornified
debris.

25 RHJ/LE Hairless ("Rhino") male mice, 5-7 weeks of
age, were obtained from Jackson Laboratories (Bar
Harbor, Maine), and treated as described in Mezick et
al., "Topical and Systemic Effects of Retinoids on Horn-
Filled Utricle Size in the Rhino Mouse: A Model to
30 Quantify "Antikeratinizing" Effects of Retinoids," 83 J.
Invest. Dermatol. 110-113 (1984) ("Mezick"), which is
incorporated herein by reference in its entirety.

EXAMPLE 2: Preparation of Topically Active Compositions

-13-

5 A sufficient amount of lyophilized trypsin,
available from Sigma-Aldrich Corporation (St. Louis,
Missouri), was mixed into a buffered aqueous solution of
0.05 M N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic
acid available from Life Technologies, Inc.
10 (Gaithersburg, Maryland) under the tradename "Hepes"
such that the pH of the resulting solution was about 7.4
and the concentration of trypsin in the solution was
about 2% (w/v). One volume of the resulting trypsin
solution was then mixed with one volume of (5%) glycerol
15 dilaurate / cholesterol / polyoxyethylene-10-stearyl
ether liposomes in water, which was prepared by the
methods described in Niemiec, in order to yield a 1%
(w/v) concentration of trypsin in the resulting
topically active composition. The glycerol dilaurate
20 was available from International Specialty Products Van
Dyke (Belleville, New Jersey) under the tradename
"Emulsynt GDL." The cholesterol was available from
Croda, Inc. (Parsippany, New Jersey) under the tradename
"Cholesterol VSP/NF." The polyoxyethylene-10-stearyl
25 ether was available from ICI Surfactants Americas
(Wilmington, Delaware) under the tradename "Brij 76."
The volume to volume ratio of trypsin to GDL liposome,
respectively, was altered to produce various
concentrations of trypsin liposomal compositions.
30 Retinoic acid compositions contained an
ethanol/propylene glycol vehicle which comprised 70%
(w/v) ethanol (ethyl alcohol, 200 proof) which was
obtained from Quantum Chemicals Corporation (Tuscola,
Illinois) and 30% (w/v) propylene glycol which was

-14-

5 obtained from Fisher Scientific (Pittsburgh,
Pennsylvania). The all-trans retinoic acid used in the
retinoic acid compositions was obtained from BASF
Aktiengesellschaft (Ludwigshafen, Germany). The volume
to volume ratio of all-trans retinoic acid to
10 ethanol/propylene glycol vehicle, respectively, was
altered to produce various concentrations of retinoic
acid compositions.

EXAMPLE 3: Delivery of Trypsin into Hair Follicles

15 About 100 μ L of the topically active trypsin
composition of Example 2 was applied to the dorsal side
of each Rhino mouse of Example 1. The trypsin used in
this composition was fluorescently-labeled with a
protein fluorescent labeling kit available from
20 Molecular Probes, Inc. in accordance with its
accompanying protocol (1996). At one and four hours
after the application of the fluorescent trypsin
treatment, a 1 cm by 2 cm sample of the skin surface of
each mouse was isolated from each mouse with scissors,
25 fixed with a 10% buffered formalin solution having a pH
of about 6.9 - 7.1 at 25°C available from Stephens
Scientific, then formed into a paraffin block according
to well-known procedures, and examined with fluorescent
microscopy according to well-known methods.

30 As shown in FIG. 1(a), almost all of the
fluorescent labeling was found within the utriculi and
sebaceous glands. The mice examined at the 1 hour
interval (FIG. 1(a)) and the 4 hour interval (FIG. 1(b))

-15-

5 displayed identical histological staining patterns, with
no additional skin penetration at the later time point.

This observation suggests against a possible non-
specific extracellular matrix digestion by the protease,
which would have likely shown a deeper penetration of
10 the fluorescent stain into the stratum corneum at the
later time point.

This Example was repeated on similar Rhino mice of
Example 1, with the exception that these mice were
treated daily for 5 days with the 1% (w/v) trypsin
15 composition of Example 2 prior to the fluorescent
trypsin treatment. Four hours after the application of
the fluorescent trypsin treatment on the fifth day, the
skins of these mice were analyzed using similar
fluorescent microscopic methods. As illustrated in FIG.
20 1(c), no major change was observed in the delivery route
of the trypsin into the utriculi and sebaceous glands of
the treated skins. However, the minimal staining at the
outer portion of the stratum corneum of the trypsin-
treated skins indicated some loss of barrier integrity.

25 This loss of barrier integrity is reflected in the
values for transepidermal water loss ("TEWL") as
described in Example 4 and Table 1 herein.

This Example shows that the application of a
topically active composition containing trypsin to the
30 skin surface of Rhino mice resulted in the delivery of
the trypsin primarily to the utriculi and sebaceous
glands, both after short term and long term use.

-16-

5 EXAMPLE 4: Trypsin Treatment Reduces the Size of
 Utricoli
 but Does Not Induce Dermal Irritation

10 Rhino mice of Example 1 were topically treated with
 the trypsin compositions (0.001% (w/v) - 1% (w/v)) of
 Example 2 once daily for five days. Animals were
 sacrificed at day 8 and image analysis was used to
 quantify the reduction in utriculi size. For image
 analysis, whole mount epidermis was processed and
 microscopic measurements were taken according to the
15 methods described in Mezick as well as Bernerd et al.,
 "The Rhino Mouse Model: The Effects of Topically Applied
 All-Trans Retinoic Acid and CD271 on the Fine Structure
 of the Epidermis and Urticuli Wall of Pseudocomedones,"
 283(2) Arch. Dermatol. Res. 100-107 (1991) and Bouclier
20 et al., "Quantification of Epidermal Histological
 Changes Induced by Topical Retinoids and CD271 in the
 Rhino Mouse Model Using a Standardizing Image Analysis
 Technique," 4(2) Skin Pharmacol. 65-73 (1991) which are
 each incorporated herein by reference in their entirety.

25 Empire Imagins Database version 1.1 was used on a
 Gateway 2000 P5-100 computer for capturing images.
 Image Pro Plus version 1.3 was used for measurements and
 Microsoft Excel version 5.0 for data processing. The
 mean utriculus diameter (μ) and the mean sebaceous gland
30 size (μ^2) were calculated for each treatment group (3
 Rhino mice), using 5 random fields, two measurements per
 field, per animal. Percent reduction in utriculi
 diameter was calculated in accordance with the methods
 described in Finney, D.J., "Parallel Line Assays,

-17-

Statistical Method in Biological Assay," Charles Griffen & Company Ltd. 69-104 (1978) which is incorporated herein by reference in its entirety.

As shown in Table 1, trypsin induced a dose dependent reduction in utriculus size that reached a plateau at ~0.1% (w/v) trypsin. A further increase in trypsin concentration did not result in more than 55% reduction of utriculus size relative to liposomal control. A small reduction in utriculus diameter was observed in the liposome vehicle alone. A single trypsin (1% (w/v)) treatment had no effect on utriculus size reduction when analyzed seven days later (not shown).

Table 1: Trypsin Induces a Dose Dependent Reduction in Utriculus Size

Treatment	Utriculus Size Reduction (%) vs. Liposome Control	TEWL (g/m ² h)
Trypsin 0.001% (w/v)	26.85 ± 4.38	29.53 ± 3.68
Trypsin 0.005% (w/v)	19.58 ± 3.06	26.40 ± 1.77
Trypsin 0.01% (w/v)	33.08 ± 2.15	28.53 ± 2.18
Trypsin 0.05% (w/v)	43.84 ± 0.62	36.57 ± 2.07
Trypsin 0.1% (w/v)	50.67 ± 0.83	42.80 ± 4.33
Trypsin 0.5% (w/v)	54.31 ± 1.33	36.23 ± 1.24
Trypsin 1.0% (w/v)	54.85 ± 1.02	42.00 ± 1.14
Liposome Vehicle	13.2 ± 1.82*	19.8 ± 1.14

* Percent of utriculus size reduction of the liposome vehicle treatment was calculated relative to the untreated control

To further characterize the effect of trypsin on the Rhino mouse skin, we measured the transepidermal water loss ("TEWL") using an "Evaporimeter EPI" evaporimeter available from Servomed AB by first

-18-

5 normalizing the evaporimeter with the ambient humidity
and then placing the probe on the dorsal skin of the
test subject at which point a reading of TEWL was taken.

10 As shown in Table 1, TEWL increased in a dose
dependent manner, with a plateau reached at ~0.05% (w/v)
trypsin. This is approximately the same concentration
for the maximal reduction in utriculi diameter. There
was no correlation between TEWL increase and visual
15 irritation. The minor scaling and erythema observed
throughout these experiments were not dose dependent and
remained low even at 1% (w/v) trypsin. Furthermore, the
TEWL for trypsin treated mice was lower than that for
retinoid treatment given alone.

20 Histological analysis of untreated, liposome
control, and trypsin treated Rhino mice skins revealed
major changes in the trypsin treated skins. H&E
staining and histological analysis were performed using
standard techniques as described in Sheehand and
Hrapchak, 1980.

25 As shown in FIG. 2(b), the trypsin treated
epidermis was hyperplastic with an increase in the
number of cell layers of both the follicular epithelium
and the epidermis when compared with the untreated
epidermis shown in FIG. 2(a). Changes were observed
30 mainly at the granular layer and the stratum corneum
resulting in restored desquamation and improved skin
structure. These epidermal changes are well-
characterized markers for retinoid activity *in vivo*, and
are associated with potential clinical efficacy. To

-19-

5 further support the assertion that trypsin is unrelated to dermal irritation, FIG.2(b) shows no inflammatory cells, which would normally be present in an irritation situation.

10 This example shows that trypsin causes a dose dependent reduction in the size of utriculi. A reduction in the size of the utriculi is associated with potential clinical efficacy of compositions for treating Acne Vulgaris. Therefore, this example further shows that trypsin is effective in the treatment of Acne
15 Vulgaris. This example further shows that topical trypsin treatments do not induce skin irritation.

EXAMPLE 5: Trypsin Treatment Results in Increased Skin Elasticity

20 Rhino mice of Example 1 which were treated with the trypsin composition of Example 2 showed a noticeable effect in skin elasticity. To quantitate this effect, a cutometer analysis was performed. We used a cutometer available from Acaderm (Menlo Park, California), and
25 employed the methods described in Couturaud et al., "Skin Biomechanical Properties: In Vivo Evaluation of Influence of Age and Body Site by a Non-Invasive Method," 1 Skin Res. and Technol. 68-73 (1995) and
30 Elsnier et al., "Mechanical Properties of Human Forearm and Vulvar Skin," 122 Br. J. Dermatol. 607-614 (1990) which are both incorporated herein by reference in their entirety. Suction was applied through a 2 mm aperture and the corresponding skin displacement and recovery after release of the negative pressure were measured.

-20-

In human studies, an improvement in the ratios of deformation parameters Ua/Uf (skin fatigue, or total recovery from the load), Ur/Uf (biological elasticity, or elastic recovery after loading), and Ur/Ue (firmness, or improvement in the deformation resistance of the skin) indicates better tonicity and elasticity of the skin. The deformation parameters Ue, Uf, Ua, and Ur are dependent, in part, on skin thickness. Consequently, ratios were used for evaluation as described in Barel et al., "Suction Method for Measurement of Skin Mechanical Properties: The Cutometer," Handbook of Non-Invasive Methods and the Skin 335-340 (1995) which is incorporated herein by reference in its entirety.

As shown in Table 2, trypsin treatment resulted in an increase in all of these parameters, which reflects improved skin elasticity. While variations between animals were significant, the increase in cutometric properties was consistent, and increased with time and length of treatment.

Table 2: Mechanical Properties of Trypsin Treated Rhino Skin

Biophysical Parameter	Day 7		Day 12		Day 16	
	Untreated Control	Trypsin Treated	Untreated Control	Trypsin Treated	Untreated Control	Trypsin Treated
Ua/Uf	0.541±0.40	0.593±0.09	0.656±0.08	0.663±0.10	0.429±0.09	0.675±0.03
Ur/Ue	0.408±0.80	0.557±0.21	0.242±0.06	0.666±0.24	0.243±0.06	0.733±0.18
Ur/Uf	0.300±0.19	0.359±0.22	0.370±0.05	0.548±0.11	0.204±0.31	0.404±0.08

-21-

5

10

To further study this elasticity effect, skin sections of Rhino mice from Example 1 treated with the trypsin composition of Example 2 were stained for elastin on paraffin sections in accordance with the methods set forth in Kligman, L.H., "Luna's Technique, A Beautiful Stain for Elastin," 3(2) The Amer. J. of Dermatopathol. 199-200 (1981) which is incorporated herein by reference in its entirety.

15

As shown in FIG. 3(b), elastin fibers (stained purple) were increased in thickness and density around the utriculi and the sebaceous glands of the trypsin treated Rhino mice when compared to the untreated mice of FIG. 3(a).

20

This same experiment was performed with C57Bl/6 mice which were obtained from Charles River Laboratories (Kingston, New York) with similar results. FIGS. 3(c) and (d), the untreated and trypsin treated skins, respectively, show the results of elastin staining. Table 3 below shows the increase in skin mechanical parameters following the trypsin treatment.

25

**Table 3: Mechanical Properties of
Trypsin Treated C57Bl/6 Skin**

Biophysical Parameter	Day 16	
	Untreated Control	Trypsin Treated
Ua/Uf	0.429±0.14	0.675±0.18
Ur/Ue	0.243±0.21	0.7335±0.02

-22-

Ur/Uf 0.204±0. 0.404±0.
 26 1

This example shows that topical treatment with trypsin increases the elasticity of C57Bl/6 and Rhino mouse skins. Skin elasticity is a property associated with anti-aging. Therefore, this example further shows that trypsin imparts anti-aging effects to the surface of the skin.

EXAMPLE 6: Trypsin Acts with a Mechanism Different from that of Retinoic Acid

The possible effect of trypsin on the sebaceous component of acne was evaluated using the hamster ear model system. Young golden Syrian hamsters, 45-55 grams upon arrival, were purchased from Charles River Laboratories (Wilmington, Massachusetts). The ventral side of the hamsters' right ears were treated daily with 10 µl of the trypsin composition of Example 2, five days a week for three weeks, while the left ears were used as untreated controls. As shown in Table 3, trypsin had no effect on the size of the sebaceous gland in this system.

Table 3: Effect of Trypsin on Size of Hamster Ear Sebaceous Gland

Treatment	Sebaceous Gland Size (µ ²)	Percent Size Decrease (%)
Untreated	99112.4 ± 2904.0	N/A
Liposome Vehicle	94698.9 ± 4997.1	4.45 (vs. untreated)
Trypsin 0.5% (w/v)	95043.0 ± 4269.1	-0.36 (vs. liposome)

-23-

vehicle)

5 This example shows that trypsin had no effect on the size of the sebaceous glands in the hamster ear model system. It is well known that in this type of model, retinoids induce a dose dependent reduction in the size of the hamster ear sebaceous glands.

10 Therefore, this example further suggests that trypsin functions with a mechanism different from that of retinoid compounds.

15 EXAMPLE 7: Trypsin and Retinoic Acid Exhibit an Additive Therapeutic Effect

A first set of Rhino mice of Example 1 were treated with suboptimal doses of the trypsin composition of Example 2. As used herein, "suboptimal" is defined as levels of trypsin concentration below the optimum for utriculi size reduction as demonstrated in Example 4. A second set of Rhino mice of Example 1 were treated with suboptimal concentrations of the all-trans retinoic acid composition of Example 2. A third set of Rhino mice of Example 1 were treated with both suboptimal doses of the trypsin composition of Example 2 and the all-trans retinoic acid composition of Example 2. In this third set of Rhino mice, the trypsin and all-trans retinoic acid treatments were each administered daily, but at different times (i.e. trypsin in the morning and all-trans retinoic acid in the afternoon). Mice were sacrificed and their skins were examined histologically with the procedure set forth in Example 3.

-24-

5 As shown in FIG. 4(c), Rhino mice treated with both
the trypsin and all-trans retinoic acid compositions
showed much improved desquamation when compared to the
trypsin and all-trans retinoic acid treatments given
10 alone (FIGS. 4(a&b)), though the treatments given alone
showed marked improvement over the untreated skin (FIG.
2(a)). Furthermore, the histological analysis revealed
far fewer open utriculi in the surface of treated skins
than either treatment given alone.

15 This example shows that a combined treatment of
trypsin and all-trans retinoic acid produces an additive
effect on skin surface characteristics such as the
number of open utriculi, which means that these
compositions are effective in the treatment of Acne
Vulgaris.

20 **EXAMPLE 8: Trypsin Eliminates PCD in the Follicular
Epithelium**

Rhino mice of Example 1 were treated daily with a
0.1% (w/v) trypsin composition of Example 2 for five
25 days and sacrificed at day eight.

1 cm by 2 cm samples of the skins of untreated,
vehicle treated, and trypsin treated mice were obtained
via the procedure set forth in Example 3 then analyzed
using a TdT-mediated dUTP-biotin nick end labeling
30 ("TUNEL") stain procedure as disclosed in Gavrieli et
al., "Identification of Programmed Cell Death in situ
Via Specific Labeling of Nuclear DNA Fragmentation", 119
Jl. Cell Biology 493-501 (1992) ("Gavrieli"). During
this procedure, the prepared skin sections were stained

-25-

5 using an "ApopTag TM Plus In Situ Apoptosis Detection
Kit" available from Oncor, Inc. as specified in the
"ApopTag TM Plus In Situ Apoptosis Detection Kit"
protocol by Oncor, Inc. (Feb. 1995), which is based upon
the labeling of fragmented DNA ends as described in
10 Gavrieli. FIGS. 5(a-d) show a histological analysis
wherein the stain has a peroxidase end point (brown) and
a methyl green counter-stain. The resulting
representations of this are provided in FIGS. 5(a&b)
which are vehicle treated and FIGS. 5(c&d) which are
15 trypsin treated.

As illustrated in FIGS. 5(a-d), the TUNEL-stained
samples defined apoptotic cells by both morphology
(condensed or fragmented nuclei and cytoplasm or
apoptotic bodies) and by the color of its stain
20 (fragmented DNA within the condensed nuclei were stained
brown). As shown in FIGS. 5(a&b), TUNEL staining
revealed an unusually high level of apoptotic bodies in
the follicular epithelium. Trypsin treatment resulted
in the elimination of all the apoptotic bodies within
25 the follicular epithelium and the restoration of
programmed cell death ("PCD") at the granular layer
(FIGS. 5(c&d)) as epidermal differentiation was
restored.

This example suggests that trypsin could restore
30 the balance between cell death and proliferation within
the follicular epithelium and within the epidermis. One
of the contributing pathological processes of Acne
Vulgaris is hyperkeratinization, which may result from a
shift in this balance. Therefore, this example further

-26-

5 shows that the ability of trypsin to restore the proper balance in epithelial cell death and proliferation may be a factor in its ability to treat Acne Vulgaris.

EXAMPLE 9: Trypsin Induces Changes in Gene Expression

10 Rhino mice of Example 1 were treated daily with trypsin compositions (0% (w/v), 0.0001% (w/v), 0.001% (w/v), and 0.01% (w/v)), as prepared in Example 2, for five days and sacrificed at day eight. The skins of vehicle treated mice and trypsin-treated mice were
15 obtained as described in Example 3, then their total RNAs were extracted using "RNA Stat-60" reagent available from Tel-Test "B," Inc. as described in Chomczynski, "Single Step Method of RNA Isolation By Acid Guanidinium Thiocyanate-phenol-chloroform
20 extraction," 162 Anal. Biochem. 156-59 (1987) which is incorporated herein by reference in its entirety. A sufficient amount of RNase-free DNase available from Promega, Corp. under the tradename "RQ1 RNase-free DNase" was then added to the extracted RNA from each
25 mouse such that each respective product contained 200 ng of DNased-RNA using the procedure set forth in "RNase-free DNase" protocol published by Promega, Corp. (May, 1995). The resulting 200 ng of DNased-RNA was reverse transcribed ("RT") using the procedure set forth in
30 "Superscript II Reverse Transcriptase" a protocol published by Gibco-BRL (now Life Technologies, Inc.) (April 1992), using random hexamers as random primers which are commercially available from Life Technologies, Inc.

-27-

The resulting RT products were then amplified via a polymerase chain reaction ("PCR") using about a 0.5 unit (per 100 μ l reaction) of a thermostable DNA polymerase which is commercially available from Perkin-Elmer-Cetus Corporation under the tradename "Taq polymerase," and about 0.1 μ mol/reaction of mouse glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) primers available from Clontech Laboratories, Inc. ("Clontech"), or primers as set forth in Table 4 (using the conditions in Table 4 or in accordance with the procedures set forth in the protocol accompanying the primers from Clontech).

Table 5 illustrates some of the DNA primers used, the amount of $MgCl_2$ required for the PCR reaction, and the length of the PCR cycle. Involucrin primers were as described in Marthinuss, et al., "Apoptosis in Pam212, an Epidermal Keratinocyte Cell Line: A Possible Role for bcl-2 in Epidermal Differentiation", 6 Cell Growth Diff. 239-250 (1995) which is incorporated herein by reference in its entirety.

Table 4: DNA Primers Utilized in RT-PCR Assay

DNA Primer (See attached Sequence Listing)	$MgCl_2$ (mM)	Cycle (min) @ $^{\circ}C$	Number of cycles	Seq. ID No.
Transglutaminase sense 5' AACCCCAAGT TCCTGAAG	2.5	1 @ 94; 2 @ 55; 3 @ 72	35	1
Transglutaminase antisense 5' TTTGTGCTGG GCCACTTC	2.5	1 @ 94; 2 @ 45; 3 @ 72	35	2
Elastin sense 5' TAAGGCAGCC AAATATGGTG	5	1 @ 94; 2 @ 45; 3 @ 72	35	3
Elastin antisense 5' ACCTGGATAA ATGGGAGAAA G	5	1 @ 94; 2 @ 55;	35	4

-28-

3 @ 72

5 When necessary for better visualization, the
resulting PCR products were precipitated with ethanol
according to well-known procedures. When primers for
10 G3PDH were used, only 10% of the PCR reaction products
were used.

The PCR products were then analyzed on 2%
agarose/ethidium bromide gels according to methods well-
known in the art in order to compare the level of
expression of certain genes in skins of trypsin-treated
15 and untreated mice. An RNA sample from the skin of a
Rhino mouse that was not reverse-transcribed was used as
a negative control for each PCR amplification. An RNA
sample from the skin of a six month old Rhino mouse was
used as a positive control when positive controls were
20 not commercially available. The results of the gel
analysis showed that the migration of the RT-PCR
products on the gels was always identical to that of the
positive controls, and to that of the reported amplicon
sizes.

25 The relative quality of each respective RT-PCR
reaction product was then compared by analyzing the mRNA
level of G3PDH, a "housekeeping" gene, in each
respective product. As illustrated in FIG. 6, G3PDH
gene expression was found to be similar in all samples
30 examined, which thereby enabled the analysis of the
relative levels of gene expression for the desired
genes.

-29-

5 Transglutaminase, an enzyme involved in the cross
linking and formation of apoptotic bodies, displayed
high mRNA levels in control animals, and was reduced to
below detection level with increasing concentrations of
trypsin. This shows that trypsin restored utriculi
10 homeostasis and eliminated abnormally high levels of
apoptosis in the follicular epithelium.

Elastin mRNA increased following treatment with
increasing concentrations of trypsin. Therefore, new
elastin is expressed following trypsin treatment which
15 results in increased skin elasticity, as described in
Example 5.

The level of involucrin, a marker of epidermal
differentiation, was increased following trypsin
treatment in a dose dependent manner. This indicates
20 that normal epidermal turnover and differentiation were
restored. Thus, trypsin restores the balance of
epidermal differentiation as shown in Example 8.

This Example showed that the effect of trypsin on
Acne Vulgaris and its anti-aging abilities may be
25 understood by examination of the expression pattern of a
series of genes over a range of trypsin concentrations.

Trypsin-induced changes in mRNA levels were clearly
evidenced, indicating a regulatory role for trypsin in
PCD, apoptosis, elastin expression, and epidermal
30 differentiation.

**EXAMPLE 10: Use of Compositions Containing Trypsin and
All-Trans Retinoic Acid**

-30-

5 Glycerol dilaurate/cholesterol/polyoxyethylene-10-
stearyl ether liposomes are prepared in accordance with
the procedures set forth in Niemiec, wherein the
constituent compounds of the liposomes are in a ratio of
10 about 58:15:27, respectively. Prior to mixing the lipid
and water phases to form the liposomes of Niemiec, 0.1%
(w/v) ascorbic acid is added to the water phase, and the
ingredients listed in Table 5 are added to the lipid
phase of the composition. The final pH of this
composition is adjusted to a range of 4 to 7, and
15 preferably from 4.5 to 5.5 with a suitable buffer.

Table 5: Ingredients Added to the Lipid Phase

Ingredient	% (w/v)
Tretinoin	0.01
Methyl Paraben	0.10
Propyl Paraben	0.02
Butylated Hydroxytoluene	0.05

20 A second composition, which comprises 1.0g trypsin
dissolved in a 0.05M Hepes buffer, at pH 7.4 (q.s. to
100ml), is added to the liposome composition in a ratio
of about 1 part of the second composition for every 8
parts of the liposome composition. This final
composition is suitable for immediate topical
application.

-31-

5 We claim:

1. A method for treating Acne Vulgaris and/or for producing anti-aging effects on the surface of the skin comprising topically applying to the skin of a mammal an effective amount of a topically active composition
10 comprising a first topically active agent.
2. The method of claim 1 wherein the first topically active agent is a protease.
3. The method of claim 2 wherein the first topically active agent is a serine protease.
- 15 4. The method of claim 3 wherein the first topically active agent is selected from trypsin, tryptase, carboxypeptidase-Y, protease IV, subtilysin or mixtures thereof.
- 20 5. The method of claim 4 wherein the first topically active agent is trypsin.
6. The method of claim 5 wherein the first topically active agent is present in an amount, based upon the total volume of the topically active composition, of from about 0% (w/v) to 5% (w/v).
- 25 7. The method of claim 6 wherein the first topically active agent is present in an amount, based upon the total volume of the topically active composition, of from about 0.01% (w/v) to about 1% (w/v).
8. The method of claim 1 wherein said topically active composition further comprises a pharmaceutically or
30 cosmetically acceptable vehicle.
9. The method of claim 8 wherein said pharmaceutically or cosmetically acceptable vehicle is a liposome or mixture thereof.

-32-

5 10. The method of claim 9 wherein said liposome is non-ionic.

11. The method of claim 10 wherein said liposome is comprised of:

10 a) glycerol dilaurate, glycerol distearate, or a mixture thereof;

 b) cholesterol, or a compound having a steroid backbone as found in cholesterol or a mixture thereof; and

15 c) a fatty acid ether having from about 12 to about 18 carbon atoms or a mixture thereof.

12. The method of claim 11 wherein said liposome is comprised of:

 a) glycerol dilaurate;

 b) cholesterol; and

20 c) polyoxyethylene-10-stearyl ether.

13. The method of claim 11 wherein the components of said liposome are present in a ratio of about 53:10:22 to about 63:20:32, respectively.

25 14. The method of claim 8 wherein said pharmaceutically or cosmetically acceptable vehicle is present in an amount, based upon the total volume of said topically active composition, of from about 0 mg/mL to about 100 mg/mL.

30 15. The method of claim 1 wherein the composition further comprises other ingredients such as moisturizers, cosmetic adjuvants, anti-oxidants, surfactants, foaming agents, conditioners, humectants, fragrances, viscosifiers, buffering agents, sunscreens, colorants, preservatives, and the like.

-33-

5 16. A method for treating Acne Vulgaris and/or for producing anti-aging effects on the surface of the skin comprising topically applying to the skin of a mammal an effective amount of:

- 10 a) a first topically active agent; and
 b) an effective amount of a second topically active agent.

17. The method of claim 16 wherein the first topically active agent is a protease.

15 18. The method of claim 17 wherein the first topically active agent is a serine protease.

19. The method of claim 18 wherein the first topically active agent is selected from trypsin, tryptase, carboxypeptidase-Y, protease IV, subtilysin or mixtures thereof.

20 20. The method of claim 19 wherein the first topically active agent is trypsin.

21. The method of claim 20 wherein the first topically active agent is present in an amount of from about 0% (w/v) to 5% (w/v).

25 22. The method of claim 21 wherein the first topically active agent is present in an amount of from about 0.01% (w/v) to about 1% (w/v).

23. The method of claim 16 wherein said second topically active agent is a retinoid.

30 24. The method of claim 23 wherein said second topically active agent is selected from retinoic acids, vitamin A alcohol, vitamin A aldehyde, retinyl acetate, retinyl palmitate, or other derivatives, analogs or mixtures thereof.

-34-

- 5 25. The method of claim 24 wherein said second topically active agent is all-trans retinoic acid.
26. The method of claim 24 wherein the second topically active agent is present in an amount of from about 0.0001% (w/v) to about 0.5% (w/v).
- 10 27. The method of claim 26 wherein the second topically active agent is present in an amount of from about 0.001% (w/v) to about 0.025% (w/v).
28. The method of claim 16 further comprising a pharmaceutically or cosmetically acceptable vehicle.
- 15 29. The method of claim 28 wherein said pharmaceutically or cosmetically acceptable vehicle is a liposome or mixture thereof.
30. The method of claim 29 wherein said liposome is non-ionic.
- 20 31. The method of claim 30 wherein said liposome is comprised of:
- a) glycerol dilaurate, glycerol distearate, or a mixture thereof;
 - b) cholesterol, or a compound having a steroid backbone as found in cholesterol or a mixture thereof; and
 - c) a fatty acid ether having from about 12 to about 18 carbon atoms or a mixture thereof.
- 25 32. The method of claim 31 wherein said liposome is comprised of:
- 30
- a) glycerol dilaurate;
 - b) cholesterol; and
 - c) polyoxyethylene-10-stearyl ether.

-35-

5 33. The method of claim 31 wherein the components of said liposome are present in a ratio of about 53:10:22 to about 63:20:32, respectively.

 34. The method of claim 28 wherein said pharmaceutically or cosmetically acceptable vehicle is
10 present in an amount, based upon the total volume of said topically active composition, of from about 0 mg/mL to about 100 mg/mL.

 35. The method of claim 16 further comprising other ingredients such as moisturizers, cosmetic adjuvants,
15 anti-oxidants, surfactants, foaming agents, conditioners, humectants, fragrances, viscosifiers, buffering agents, sunscreens, colorants, preservatives, and the like.

 36. The method of claim 16 wherein the first topically
20 active agent is applied to the skin of a mammal simultaneously with the second topically active agent.

 37. The method of claim 16 wherein the first topically active agent is applied to the skin of a mammal at a
25 time other than simultaneously with the second topically active agent.

 38. A pharmaceutical or cosmetic composition comprising:

- a) a first topically active agent; and
- b) a second topically active agent.

30 39. The pharmaceutical or cosmetic composition of claim 38 wherein the first topically active agent is a protease.

-36-

5 40. The pharmaceutical or cosmetic composition of claim 39 wherein the first topically active agent is a serine protease.

10 41. The pharmaceutical or cosmetic composition of claim 40 wherein the first topically active agent is selected from trypsin, carboxypeptidase-Y, protease IV, subtilysin or mixtures thereof.

42. The pharmaceutical or cosmetic composition of claim 41 wherein the first topically active agent is trypsin.

15 43. The pharmaceutical or cosmetic composition of claim 42 wherein the first topically active agent is present in an amount, based upon the total volume of the topically active composition, of from about 0% (w/v) to 5% (w/v).

20 44. The pharmaceutical or cosmetic composition of claim 43 wherein the first topically active agent is present in an amount, based upon the total volume of the topically active composition, of from about 0.01% (w/v) to about 1% (w/v).

25 45. The pharmaceutical or cosmetic composition of claim 38 wherein said second topically active agent is a retinoid.

30 46. The pharmaceutical or cosmetic composition of claim 45 wherein said second topically active agent is selected from retinoic acids, vitamin A alcohol, vitamin A aldehyde, retinyl acetate, retinyl palmitate, or other derivatives, analogs or mixtures thereof.

47. The pharmaceutical or cosmetic composition of claim 46 wherein said second topically active agent is all-trans retinoic acid.

-37-

5 48. The pharmaceutical or cosmetic composition of claim
46 wherein the second topically active agent is present
in an amount, based upon the total volume of the
topically active composition, of from about 0.0001%
(w/v) to about 0.5% (w/v).

10 49. The pharmaceutical or cosmetic composition of claim
48 wherein the second topically active agent is present
in an amount, based upon the total volume of the
topically active composition, of from about 0.001% (w/v)
to about 0.025% (w/v).

15 50. The pharmaceutical or cosmetic composition of claim
48 wherein said topically active composition further
comprises a pharmaceutically or cosmetically acceptable
vehicle.

20 51. The pharmaceutical or cosmetic composition of claim
50 wherein said pharmaceutically or cosmetically
acceptable vehicle is a liposome or mixture thereof.

52. The pharmaceutical or cosmetic composition of claim
51 wherein said liposome is non-ionic.

25 53. The pharmaceutical or cosmetic composition of
claim 52 wherein said liposome is comprised of:

 a) glycerol dilaurate, glycerol distearate, or a
mixture thereof;

 b) cholesterol, or a compound having a steroid
backbone as found in cholesterol or a mixture thereof;
30 and

 c) a fatty acid ether having from about 12 to about
18 carbon atoms or a mixture thereof.

54. The pharmaceutical or cosmetic composition of claim
53 wherein said liposome is comprised of:

-38-

- 5 a) glycerol dilaurate;
 b) cholesterol; and
 c) polyoxyethylene-10-stearyl ether.

10 55. The pharmaceutical or cosmetic composition of claim
53 wherein the components of said liposome are present
in a ratio of about 53:10:22 to about 63:20:32,
respectively.

15 56. The pharmaceutical or cosmetic composition of
claim 50 wherein said pharmaceutically or cosmetically
acceptable vehicle is present in an amount, based upon
the total volume of said topically active composition,
of from about 0 mg/mL to about 100 mg/mL.

20 57. The pharmaceutical or cosmetic composition of claim
38 wherein the composition further comprises other
ingredients such as moisturizers, cosmetic adjuvants,
anti-oxidants, surfactants, foaming agents,
conditioners, humectants, fragrances, viscosifiers,
buffering agents, sunscreens, colorants, preservatives,
and the like.

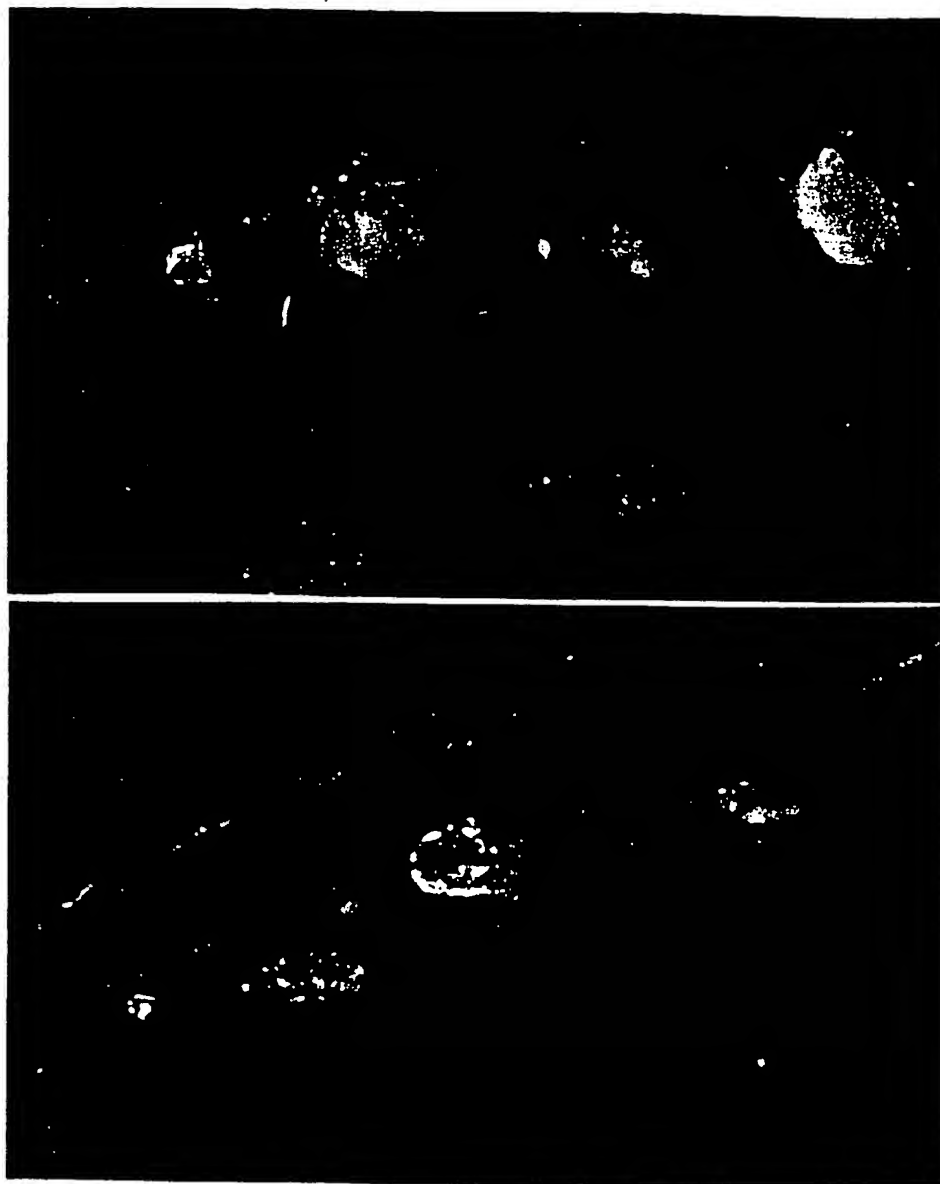
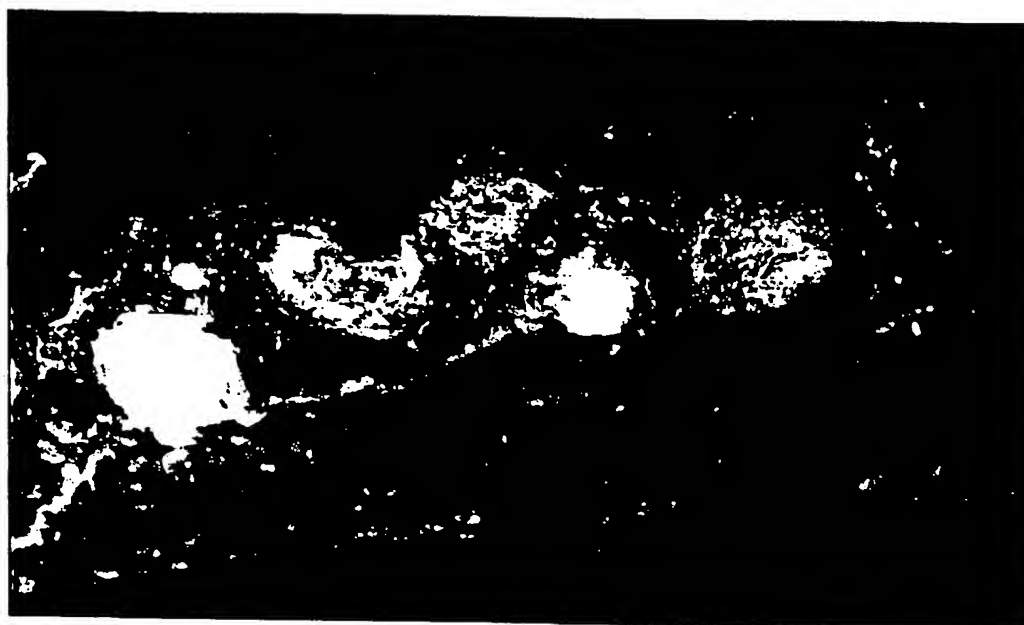


Fig 1

FIG. 1B



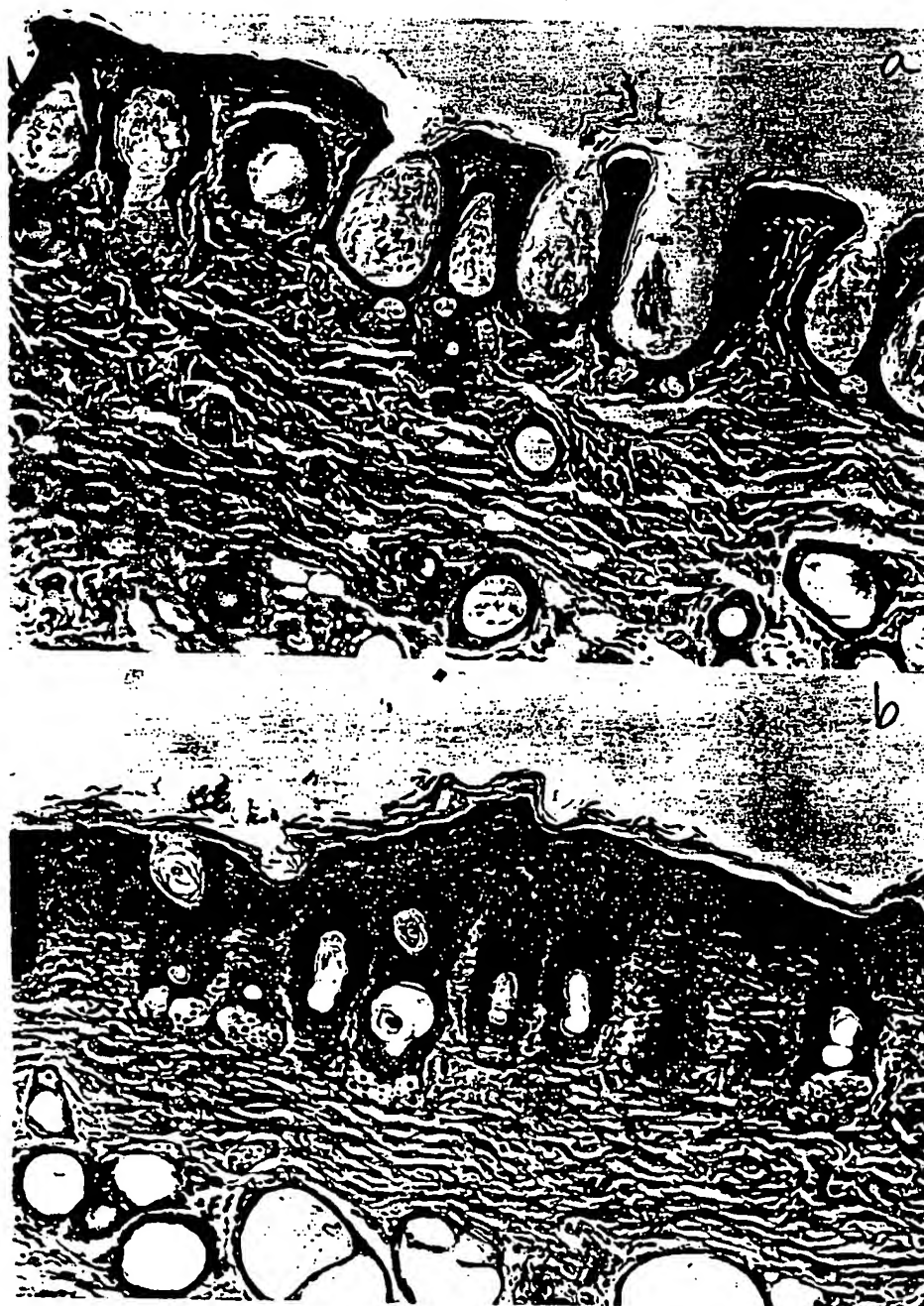


Fig 2

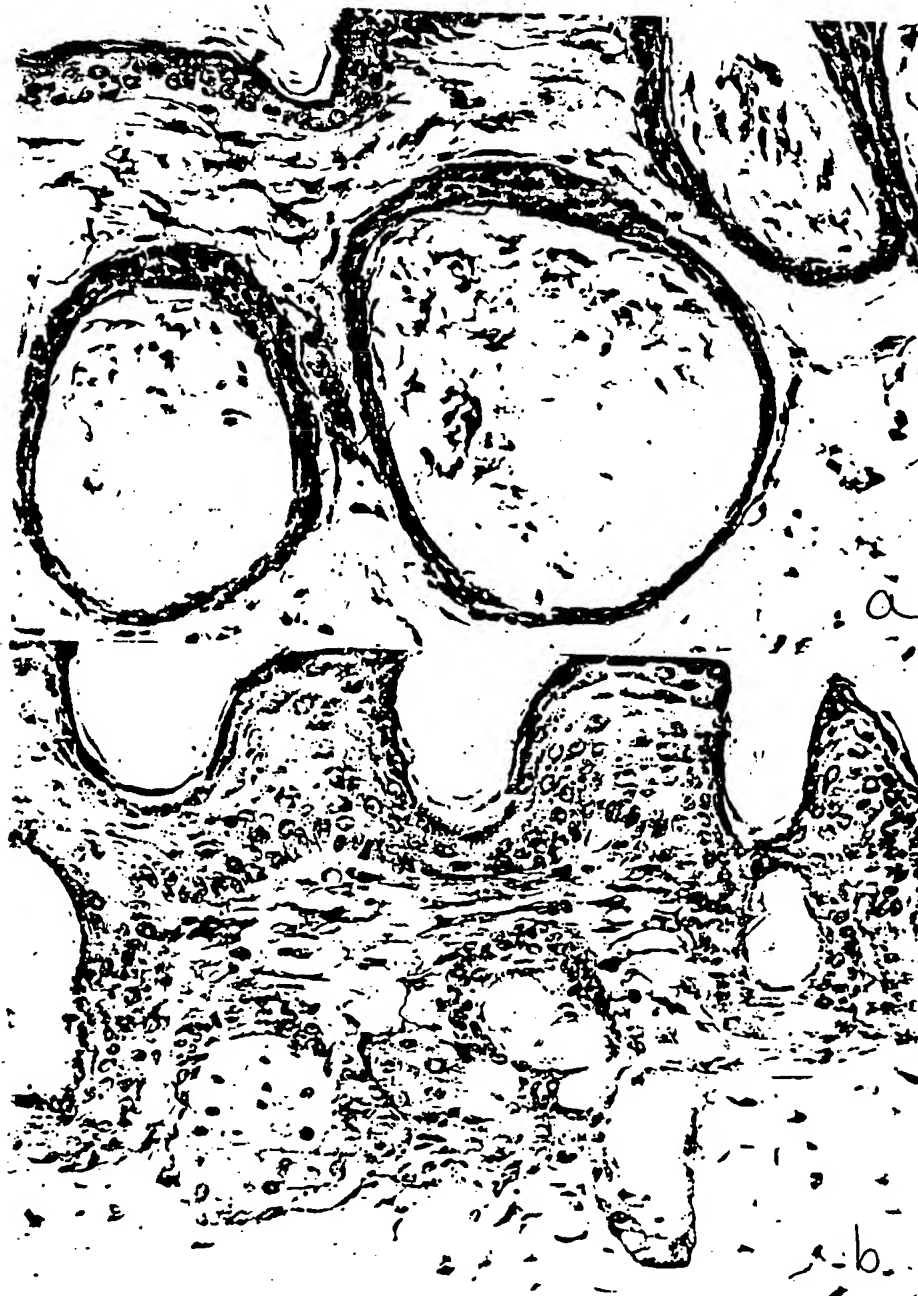


Fig 3

FIG. 3C

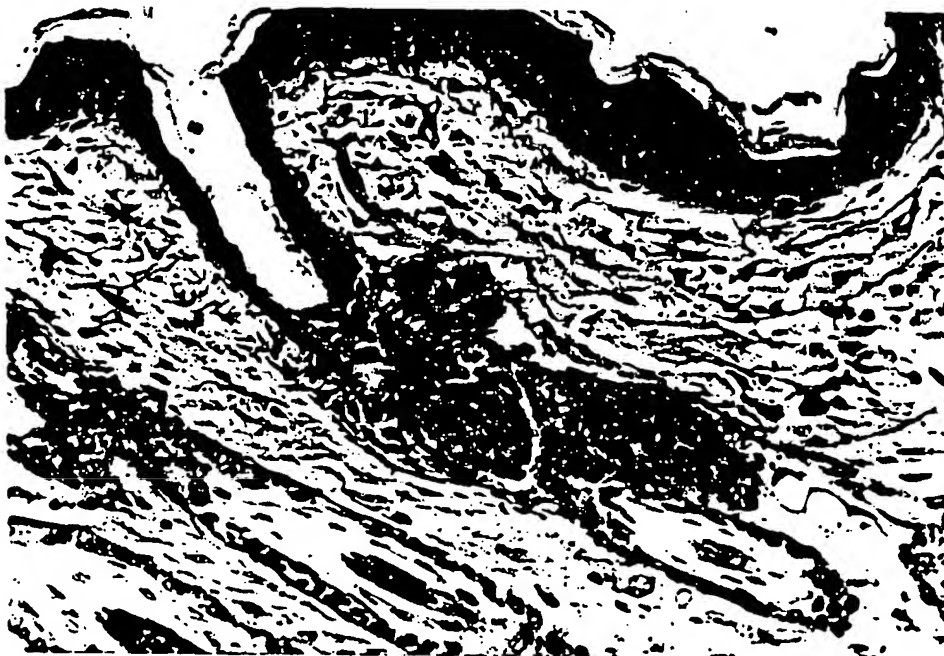


FIG. 3D

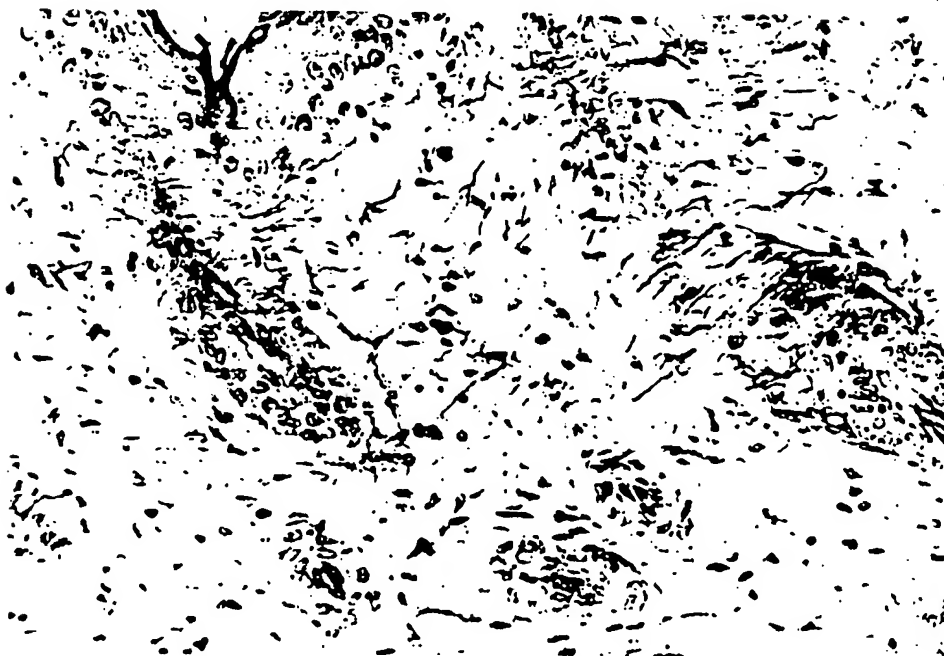




FIG 4



FIG 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/02618

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K7/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 97 25059 A (BEIERSDORF) 17 July 1997 see page 1, line 1 - page 2, line 16; claims 1,2; examples 1,2 see page 6, line 1 - page 6, line 11	1-57
X,P	STN, File Supplier, Karlsruhe, DE, File XP002073067 Chemical Abstracts, Vol 127, AN=243233 see the abstract --- -/--	1-8, 14-22, 28, 34-36, 38-44,57

☒ Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/02618

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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